

## Summary.

F9 cells (embryonal carcinoma) can be induced to differentiate with retinoic acid and dibutyryl-cAMP into cells with a phenotype resembling parietal endoderm. We show that the levels of cyclin-dependent kinase inhibitor p21/WAF1/Cip1 (p21) protein and mRNA are dramatically elevated at the end of this differentiation. Clones of F9 cells stably expressing ectopic p21 revealed, upon differentiation, upregulated levels of mRNA for thrombomodulin, a parietal endoderm-specific marker. Furthermore, p21 activated the thrombomodulin promoter in transient reporter assays and the p21 mutant defective in binding to cyclin E was equally efficient in activation. The promoter activity in differentiated cells was reduced by cotransfection of p21-specific siRNA or antisense cDNA. Coexpression of p21 increased the activity of the GAL-p300(1–1303) fusion protein on the GAL sites-containing TM promoter, implying that p21 might act through a derepression of the p300 N-terminal-residing repression domain, thereby enhancing the p300 coactivator function. Whereas p21 was strictly nuclear in undifferentiated cells, a large proportion of differentiated cells had p21 localized also in the cytoplasm, a site associated with the antiapoptotic function of p21. As differentiation of F9 cells into parietal endoderm-like cells requires the cAMP signaling, the results together suggest that the cyclin-dependent kinase inhibitor p21 may promote specifically this pathway in F9 cells and may have an antiapoptotic role in these cells.

Further study investigated the expression and transcriptional activity of p21/WAF1/Cip1 in human melanoma cells in culture. p21 was abundantly expressed in melanoma cells and was expressed also in proliferating epidermal melanocytes. Clones of melanoma cells with p21 knocked-down by siRNA had even decreased growth rates. We further found that promoters of several genes related to differentiation were stimulated by p21 overexpression, including the MITF promoter. The N-terminal portion and intact cyclin-binding site was

essential for these transcriptional activities, suggesting that mechanisms other than cell cycle inhibition, PCNA binding, and derepression of p300/CBP repression domain CRD1 underlie the transcriptional effects. Since activation of the p21 gene expression by MITF was described previously, the reciprocal stimulation suggested here might represent a positive-feedback loop reinforcing the presumed prosurvival activity of MITF in melanoma cells.

Since recent data implicate MITF as a survival factor for melanoma cells, we further tested whether the melanocyte-specific MITF promoter activity can be inhibited directly by an adenoviral protein E1A and its mutants. We found strong repression by the wild type E1A protein. The two motifs which mediate E1A binding to the retinoblastoma protein or to the transcriptional co-activator TRRAP, and are important for transformation by E1A in cooperation with other oncogenes, were dispensable for repression. Thus, this transformation-defective E1A mutant might constitute a good targeting agent for antimelanoma therapy. We further used the E1A oncoprotein and its mutants as repressors of both the transiently transfected and endogenous tyrosinase promoter. We have shown that the requirement of the E1A N-terminus for repression of the MITF-activated tyrosinase promoter and the sensitivity to derepression by the histone deacetylase inhibitor trichostatin are distinct when the activity of the transiently transfected or the endogenous promoter is analysed in U2-OS cells. Thus, for transiently transfected versus chromatin-embedded promoter, the activity of obligatory MITF seems to be executed through different mechanisms of transcriptional coactivation.

Although numerous MITF-dependent downstream genes have been identified, the mechanisms by which the MITF activity is coregulated remain elusive. We show that the adenoviral E1A protein represses the MITF-driven transcription in U2-OS cells. The E1A CR1 domain (which alone is insufficient to bind p300) was sufficient for repression, while the N-terminus, through which E1A binds the p300/CBP proteins and other coactivators, was unable to repress. Correspondingly, CR1 inhibited colony formation of MITF-positive, but

not MITF-negative, melanoma cells. The repression by CR1 was largely independent of the PCAF-binding motif, previously recognized to be necessary for suppression of muscle-specific enhancer. Interestingly, CR1 conferred transcriptional competence to the MITF-CR1 chimera in which the MITF portion was rendered transcription-deficient. Moreover, MITF mutants defective in binding to p300/CBP *in vivo* still activated transcription, further supporting a p300/CBP-independent coactivation of MITF targets. Our results suggest that understanding how CR1 represses *Mitf* activity may reveal a route to melanoma therapy.

Also, a dominant negative mutant of the melanocyte-specific isoform of MITF is described carrying deletions of both N- and C-terminal transactivation domains. Cotransfection of this mutant resulted in a complete inhibition of the wild type MITF function as tested on both the reporter-linked tyrosinase promoter and an endogenous, ectopic MITF-triggered tyrosinase gene in U2-OS cells. The dominant negative construct also strongly repressed the activity of a hyperactive MITF-Vp16 chimera. Importantly, deletion of both activation domains was necessary to eliminate the residual transcription activity observed when only the N-terminal domain was removed and to achieve the repressive effect in human melanoma cells. If the activity of MITF plays a role in the long-term survival of malignant melanocytes, overexpression of a strong dominant negative MITF mutant might be a useful strategy to suppress its transactivation function.